Fragmentation Analysis of Bradykinin by ^{252}Cf -Plasma Desorption Mass Spectrometry

David M. Bunk and Ronald D. Macfarlane

Texas A & M University, Department of Chemistry, College Station, Texas, USA

The ^{252}Cf -plasma desorption (PD) mass spectrum of the nonapeptide bradykinin was studied in detail with particular emphasis on the fragmentation pattern resulting from fast metastable decay of the molecular ion. N-acetyl and O-methyl derivatives of bradykinin were used as mass shift species for assignment of N-terminal and C-terminal fragment ions. Thirty-seven sequence-specific fragment ions were identified, including those that are due to cleavage of peptide backbone as well as side chain bonds (i.e., d_n , v_n , w_n fragment ions). The degree of fragmentation correlates with what has been observed by fast atom bombardment tandem mass spectrometry using high energy collisional activation. The high degree of excitation of bradykinin molecules in ^{252}Cf -PDMS is undoubtedly due to the special nature of excitation intrinsic to the ^{252}Cf -PD process where electronic excitation occurs with a very high probability due to the high electron and photon flux surrounding the nuclear fission fragment track.

These results offer further evidence that ^{252}Cf -PDMS, in addition to providing molecular weight information, can be used to sequence peptides without the need for a second stage of molecular excitation. (*J Am Soc Mass Spectrom* 1991, 2, 379–386)

Used in the determination of the molecular weights of a wide spectrum of complex molecules of biological interest [1], more recently ^{252}Cf -plasma desorption mass spectrometry (^{252}Cf -PDMS) has been equally successful with metal cluster compounds with masses approaching 100 kD [2]. ^{252}Cf -PDMS remains as one of the most universally applicable mass spectrometric methods. One of the features of the method is its simplicity and reliability of operation, which has led to its adoption in a number of biomedical laboratories as a standard analytical instrument. A perceived limitation of the method is that little structural information can be obtained from a ^{252}Cf -PD mass spectrum. Consequently, ^{252}Cf -PDMS has been sometimes referred to as an efficient "molecular weight machine" [3]. Part of the reason for this misconception is due to the emphasis that had been placed on extending the mass range capability of ^{252}Cf -PDMS for the studies of biopolymers with increasing molecular mass. This has involved a considerable effort in improving the instrumentation and finding new adsorption/desorption matrices for enhancing the yield of high mass protein molecular ions. These activities were carried out at the expense of exploiting the features that were present in ^{252}Cf -PD mass spectra, namely, fragmentation patterns that could be correlated with structure. Even

though we recognized in our first measurements in the early days of the development of ^{252}Cf -PDMS that fragment ions were present in ^{252}Cf -PD mass spectra [4], only for a few studies in our laboratory were fragmentation patterns analyzed for the purpose of obtaining structure information.

One of the first extensive uses of fragmentation patterns generated by ^{252}Cf -PDMS for structural studies was carried out by McNeal et al. [5] in their characterization studies of synthetic oligonucleotides where an intense negative ion fragmentation pattern was observed due to the weakness of the oligonucleotide's phosphodiester bond. Chait et al. [6] were the first to utilize the fragmentation pattern in a ^{252}Cf -PD mass spectrum of a peptide to verify the sequence of synthetic alamethicin I which contains 20 amino acid residues. Fales et al. [7], in a study of polymethylenediamine-linked enkephalin peptides, showed that the ^{252}Cf -PD mass spectra of these species contained not only the peptide fragment ions, but also a complete cleavage pattern of the polymethylene link, an excellent example of "charge-remote site" fragmentation that was first recognized by Tomer et al. [8]. Roepstorff et al. [9] reported the fragmentation of the pentacyclic peptide Nisin, although the extent of fragmentation was completely dependent on the sample preparation method. Despite these earlier positive indications, the use of ^{252}Cf -PDMS for sequence determination has not been vigorously pursued and the general view of the potential for ^{252}Cf -PDMS as a

Address reprint requests to Ronald D. Macfarlane, Department of Chemistry, Texas A & M University, College Station, TX 77843-3255.

sequencing method has been unjustifiably pessimistic.

Recent studies by Vorst et al. [10] and Buko and Sarin [11] have shown that complete sequence information could be obtained from the primary ^{252}Cf -PDMS spectrum (primary in the sense that no additional fragmentation of the primary, desorbed ions is used) for native peptides up to 5.5 kD and suggest that the fragmentation is comparable to what is observed by using fast atom bombardment tandem mass spectrometry (FABMS/MS) coupled with high energy collision-induced dissociation (CID). This exciting new development has encouraged us to begin an extensive program to study the possibilities of using the primary ^{252}Cf -PD mass spectrum to sequence peptides and proteins. Our first results are reported here.

Bradykinin was the model peptide chosen for this work because its fragmentation has been extensively characterized over a wide range of excitation energies. The first objective of this study was to record the fragmentation pattern and use established protocols for resolving fragment ion peaks from those of the matrix. The second objective was to determine the excitation energy regime for ^{252}Cf -PDMS by comparison of our fragmentation patterns for bradykinin with those obtained by others from direct excitation in FAB [12], by the decay of FAB-generated metastable molecular ions [13], and by CID of FAB-generated molecular ions in the low- and high-ion velocity regime [14].

Experimental

Instrumentation

Mass spectra were recorded on a ^{252}Cf -PD mass spectrometer that was designed and built in our laboratory. Details of this system have been published [15]. A $7\mu\text{Ci}$ ^{252}Cf source (Isotope Products, Inc., Burbank, CA) was positioned 4 mm behind the sample target, giving an effective fission fragment flux through the sample of 2100/s. An acceleration voltage of +15 kV was used for positive ions, and -10 kV for negative ions. The length of the field-free region was 52 cm, and a new ion detector, consisting of two 40-mm diameter microchannel plates coupled by an electron focusing lens to a 25-mm diameter impedance matched anode, was used at the end of the flight tube. This detector gives improved sensitivity. Data accumulation time was increased to 720 min for these measurements to enhance signal/background ratios for low intensity ions.

Mass calibration of the positive PD mass spectra was accomplished using the H^+ , C_2H_3^+ , and phenylalanine immonium (m/z 120) ions. Using just the H^+ and C_2H_3^+ ions for mass calibration resulted in the observed masses for all of the molecular and fragment ions to be lower than their calculated masses [16]. We have found that incorporating the higher mass phenylalanine immonium ion into the calibration improved

the mass accuracy for all ions and resulted in mass errors that were both positive and negative in sign. The H^+ and C_2H_3^+ ions were used to calibrate the negative PD mass spectra.

Purifications and separation of bradykinin and derivatives were carried out by using a reverse phase high performance liquid chromatography (HPLC) column (Applied Biosystems Aquapore C_8 RP-300, 1×50 mm) connected to a microseparations system (Model 130A, Applied Biosystems, Foster City, CA). Chromatograms were obtained by using a data module (Model 745B, Waters, Milford, MA).

Reagents and Samples

Bradykinin (Serva, Westbury, NY) was purified just prior to use by reverse phase HPLC using the system described above. Acetic anhydride (Mallinckrodt, Paris, NY), sodium acetate and concentrated hydrochloric acid (HCl) (Fisher, Fair Lawn, NJ), and methanol (Burdick & Jackson, Muskegon, MI), as well as solvents used in the HPLC separations were used without further purification.

Sample Preparation and Synthesis

The peptide samples for PDMS were purified by reverse phase HPLC using a linear binary gradient elution with 0.1% trifluoroacetic acid (TFA) as the aqueous component and 0.1% TFA in 80% acetonitrile as the organic component. Approximately 250 pmol of peptide were injected onto the HPLC column. When the peptide fraction was eluted from the column (ca. 25 μL elution volume), it was collected directly onto a ^{252}Cf -PDMS sample holder consisting of an electro-sprayed nitrocellulose coating (100 $\mu\text{g}/\text{cm}^2$) supported on a 1.5- μm thick aluminized polyethylene terephthalate film. This is referred to in later discussions as the sample target. We collected the peptide fraction directly onto the target to minimize sample loss and reduce the possibility of introducing contaminants. The peptide fraction was then evaporated to dryness under a stream of nitrogen to ensure saturation coverage of the nitrocellulose by the peptide. A droplet of water was then applied to the surface of the peptide/nitrocellulose deposit and allowed to stand for a few seconds to redissolve water-soluble impurities and peptide that was not firmly adsorbed to the nitrocellulose. The droplet was removed by centrifugation at 8000 rpm by using a photoresist spinner (Headway Research, Garland, TX).

Acetylation of the N-terminal amine of bradykinin was carried out by using an established procedure [17]. Bradykinin (1 μmol) was dissolved in an equal volume of water and a saturated solution of sodium acetate (pH = 9.2). A hundredfold molar excess of acetic anhydride was then added to the peptide solution in four equivalent aliquots at 0 $^\circ\text{C}$ over a period of 1 hr. The reaction products was separated by HPLC,

and the singly acetylated fraction was collected on a nitrocellulose target and analyzed by ^{252}Cf -PDMS. The yield of the N-terminal acetylated bradykinin was estimated from the HPLC chromatogram to be approximately 90%. A second fraction comprising the remaining 10% was found by ^{252}Cf -PDMS to be the diacetylated peptide with the second acetyl group located on a serine residue.

To prepare the C-terminal methyl ester analog of bradykinin, 1 μmol of the peptide was dissolved in methanolic 0.01 N HCl and reacted for 12 h at 5 $^{\circ}\text{C}$ [17]. The HCl/methanol solution was prepared by adding concentrated HCl to methanol. The reaction products were separated by HPLC, collecting the desired product fraction directly on a nitrocellulose target. From the HPLC chromatogram, the yield of the bradykinin methyl ester was estimated to be 60%, with the remainder being the unreacted peptide.

Results and Discussion

General Plan

This study is part of a long-term goal to develop ^{252}Cf -PDMS as a peptide sequencing method, so we deemed it necessary to establish a general protocol to determine unequivocally which ions in the ^{252}Cf -PD mass spectrum were indeed fragment ions from bradykinin. Because we wish to use only the *primary* mass spectrum for the sequence determination, we must first distinguish background ions from the bradykinin fragment ions. The nitrocellulose substrate, which may also contain adsorbed molecules from the ambient residual gas in the mass spectrometer, is the main source of background ions. A ^{252}Cf -PD mass spectrum of the nitrocellulose target without adsorbed bradykinin gives some indication of the major substrate ion peaks in the mass region of interest that may also appear in the bradykinin spectrum. Background ions can also come from impurities in the bradykinin sample that coelute with bradykinin in the HPLC fraction and coadsorb onto the nitrocellulose. By using a high resolution microbore column in the HPLC purification, this contribution is minimized.

After an ensemble of peaks has been identified in the bradykinin spectrum as candidate fragment ions, the final criterion that a candidate fragment ion is indeed a real fragment ion is that an appropriate mass shift is observed for that peak in the mass spectrum of either the N-terminal or C-terminal derivatized peptide. This technique has been previously used to identify N-terminal and C-terminal fragment ions [18].

The Positive Ion Mass Spectrum of the Nitrocellulose Substrate in the Region of Interest

The positive ion spectrum of nitrocellulose in the mass region of interest (m/z 195-1160) recorded for a 720-min period is shown in Figure 1a. The spectrum

contains a number of prominent ions in this mass region and an assortment of ion peaks with lower intensity. The most prominent ions that persist when bradykinin is adsorbed onto the surface are at m/z 219, 280, 391, 419, and 570. No attempt has been made to identify the structure of these ions.

The Positive Ion Mass Spectrum of Bradykinin Adsorbed on Nitrocellulose

The ^{252}Cf -PD positive ion spectrum of bradykinin (RPPGFSPFR) adsorbed on nitrocellulose in the mass region m/z 195-1160 is shown in Figure 1b. The most prominent ion is the bradykinin molecular ion at m/z 1061.21 $\pm$ 0.20, a mass that is in good agreement with the isotopically averaged mass of the protonated molecular ion (calculated, 1061.23 u). The doubly protonated species was also observed at m/z 530.98 $\pm$ 0.20 (calculated, 531.12 u).

In the first analysis of the bradykinin fragmentation spectrum the primary concern was whether the background contribution from the nitrocellulose matrix would give too much interference. Fortunately, some of the ions that are due to the nitrocellulose are significantly attenuated in intensity when the bradykinin is adsorbed on the surface. For example, the prominent peaks at m/z 219, 280, and 391 are reduced in intensity by 70% when bradykinin is adsorbed, suggesting that these ions originate from adsorption/desorption sites on the nitrocellulose structure. In addition to the ions that were known to originate from the substrate, 37 structurally significant

Figure 1. Positive ion plasma desorption mass spectra of (a) nitrocellulose and (b) bradykinin adsorbed on nitrocellulose, indicating the nitrocellulose matrix peaks present in both spectra.

ions were identified as originating from fragmentation of the protonated (or, possibly, doubly protonated) molecular ion of bradykinin. The total intensity (integrated peak areas) of the fragmentation ion spectrum was found to be comparable to the sum of the intensities of the protonated and doubly protonated molecular ion. These ions are listed in Table 1.

The Positive Ion Spectra of N-acetylated and O-methylated Bradykinin Adsorbed on Nitrocellulose

The objective of this series of experiments was to determine whether the complex pattern of fragment ions observed in the native bradykinin spectrum could be resolved into the two classes of N-terminal and C-terminal fragment ions. This was accomplished by preparing N- and C-terminal derivatives of bradykinin: N-acetyl bradykinin and O-methylated bradykinin. Both derivatives were prepared by using standard methods outlined in the Experimental section, followed by purification by microbore HPLC. All peaks in the primary bradykinin spectrum that were shifted by 42 u in the spectrum for the N-acetyl derivative were assigned to N-terminal fragment ions; those that shifted in mass by 14 u for the O-methylated derivative were assigned to C-terminal fragment ions. Those peaks that did not shift for either derivative can only be due to background peaks or internal fragmentation of the peptide.

The mass spectra for bradykinin and the two derivatives have been divided into three mass regions and are shown in Figures 2-4. Figure 2 covers the mass region from m/z 195-500. Two examples of the mass shift method are depicted. The peak at m/z 254.25 in the bradykinin spectrum (Figure 2b) was not present in the spectrum of the N-acetylated derivative (Figure 2a), but was present in the spectrum of the O-methylated derivative (Figure 2c). The assignment of this peak as an N-terminal fragment ion was confirmed by the presence of a new peak in the spectrum of the N-acetylated derivative that is shifted in mass by 42 u. This peak was subsequently designated as a b_2 fragment ion (using the notation of Roepstorff and Fohlman [19], with the modifications suggested by Biemann [20]), having a calculated m/z of 254.31. In a similar manner, the peak at m/z 348.30 was identified as a C-terminal fragment ion from the observation that this peak was still present in Figure 2a, but was absent in Figure 2c with a new peak appearing 14 u higher. This peak was subsequently designated as the x_2 fragment ion (calculated m/z = 348.38). This procedure was used to identify the majority of the peaks in the bradykinin spectrum in this mass region and to identify which ions are not from bradykinin. For example, the peak m/z 279.4, present in all three spectra and in the bare nitrocellulose spectrum (Figure 1a), is clearly a peak from the background.

Table 1. Molecular and fragment ions of bradykinin

Ion	Observed mass ^a (u)	Calculated mass ^a (u)	Relative intensity ^b
(M + H) ⁺	1061.21	1061.23	1.00
(M + 2H) ²⁺	530.98	531.12	0.13
a_2	226.29	226.30	0.14
a_3	323.37	323.42	0.14
a_4	380.39	380.47	0.04
a_5	527.45	527.65	0.11
a_6	614.75	614.72	0.05
a_7	711.76	711.84	0.07
a_8	859.44 ^c	859.02	0.06
b_2	254.25	254.31	0.04
b_4	408.39	408.48	0.01
b_6	642.69	642.73	0.03
$c_3 + 2$	368.41	368.46	0.01
$c_4 + 2$	425.41	425.51	0.02
$c_5 + 2$	572.80	572.69	0.02
$c_7 + 2$	757.06	756.88	0.01
$c_8 + 2$	903.95 ^d	904.06	0.04
d_6	598.89	598.72	0.04
d_9	931.00 ^e	930.10	0.03
v_2	230.24	230.25	0.07
v_4	474.29	474.54	0.03
v_6	561.63	561.62	0.02
w_3	376.48	376.43	0.02
w_7	765.21	764.86	0.01
x_1	201.20	201.20	0.09
x_2	348.30	348.38	0.04
$x_3 + 1$	446.48	446.51	0.05
x_5	679.85	679.75	0.02
x_6	736.62	736.80	0.03
$x_7 + 1$	834.87	834.93	0.01
$x_8 + 1$	931.00 ^e	932.05	0.03
y_3	417.49	417.48	0.08
y_7	805.77	805.91	0.03
y_8	903.95 ^d	903.02	0.04
$z_1 + 1$	159.20	159.19	0.16
$z_2 + 1$	306.34	306.36	0.06
$z_4 + 1$	490.38	490.56	0.02
$z_5 + 1$	637.26	637.73	0.01
$z_6 + 1$	695.22	694.78	0.01
-R _{arg}	960.86	961.08	0.04
-R _{phe}	969.56	970.09	0.02
-R _{ser}	1030.30	1030.02	0.03
-COOH	1015.94	1016.21	0.06
-NH ₃	1043.79	1044.20	0.02

^a Isotopically averaged mass

^b Relative to bradykinin [M + H]⁺

^c $a_8/a_8 + 1$ unresolved

^d $c_8 + 2$ and y_8 ions unresolved

^e d_9 and $x_8 + 1$ ions unresolved

Figure 3 covers the mass range of m/z 500-790 for bradykinin and the two derivatives. Two more examples of fragment ion assignments based on mass shifts are graphically presented: the ion at m/z 598.89,

Figure 2. Positive ion mass spectra of (a) N-acetylated bradykinin, (b) bradykinin, and (c) O-methylated bradykinin in the mass range 195 to 500 u.

Figure 3. Positive ion mass spectra of (a) N-acetylated bradykinin, (b) bradykinin, and (c) O-methylated bradykinin in the mass range 500 to 790 u.

which was determined to be an N-terminal fragment ion and was subsequently assigned as a d_6 fragment ion (calculated $m/z = 598.72$), and an ion at m/z 679.85, a C-terminal fragment ion identified as an x_5 fragment ion (calculated $m/z = 679.75$). The intensity of the doubly protonated molecular ion that appears in this mass region is not affected by the presence of the functional groups that are added to the molecule.

Figure 4 covers the mass region from m/z 790–1160 and includes the molecular ion plus the heaviest N- and C-terminal fragments. The peak at m/z 903.95 in the native bradykinin spectrum (Figure 4b) could be assigned as either a y_8 (calculated $m/z = 903.02$) or a $c_8 + 2$ (calculated $m/z = 904.06$) ion. In the spectrum of the O-methylated bradykinin (Figure 4c), these two ions are resolved due to the 14-u mass shift of the C-terminal y_8 ion. Therefore, the m/z 903.95 peak in the native bradykinin can be attributed to unresolved y_8 and $c_8 + 2$ ions. Likewise, the m/z 931.00 peak in the native bradykinin spectrum can be assigned to both a d_9 (calculated $m/z = 930.10$) and an $x_8 + 1$ (calculated $m/z = 932.05$) ion through the appropriate mass shifts in the spectra of both derivatives.

In addition to the appearance of N-terminal and

C-terminal fragment ions, a collection of peaks appears near the mass of the molecular ion that corresponds to the loss of neutral molecules and side chains from some of the amino acid components of bradykinin. For example, weak peaks corresponding to loss of $-NH_3$ are observed for the native molecule and the methyl ester. Loss of $-COOH$ is a strong fragment ion for the parent molecule and the N-acetyl derivative but is absent for the O-methyl ester, being replaced by loss of $-COOCH_3$. Side chain losses from serine, arginine, and phenylalanine residues are also observed. A weak peak (1% of the intensity of the $[M + H]^+$ ion) was also observed at m/z 1077, which we attribute to a minor contaminant of the sample. Because of its low abundance, this contaminant does not measurably contribute to the fragmentation ion spectrum.

The results of the fragmentation pattern analysis for bradykinin including the fragment type, observed and calculated mass, and the relative intensity are summarized in Table 1. The average error in mass for the data set was determined to be -0.05 ± 0.20 u.

The fragmentation pattern for bradykinin is rich with structural information and is easily discernible

Figure 4. Positive ion mass spectra of (a) N-acetylated bradykinin, (b) bradykinin, and (c) O-methylated bradykinin in the mass range 790 to 1160 u.

from the background peaks. An inventory of the ions detected in the bradykinin spectrum shown in Figure 1b has revealed some interesting statistics. Approximately 9.5 million positive ions originating from bradykinin were detected in the 12-h acquisition that were either molecular ions or sequence-specific ions. For the molecular ion component, 47% of the total comprised the protonated molecular ion; 3% the doubly protonated ion. The remaining 50% were distributed as N-terminal fragment ions (27%), C-terminal fragment ions (13%), and fragment ions resulting from neutral molecule loss from the molecular ion (10%).

The fragmentation pattern is dominated by the $a_n/a_n + 1$ series, which forms a doublet pattern that is resolved at low mass (Figure 2b) but not at high mass (Figure 3b). Additional N-terminal fragment ions include not only representatives from the b_n and $c_n + 2$ series but also two d_n fragment ions (d_6 and d_9). The d_6 ion assignment was confirmed in the ^{252}Cf -PDMS spectrum of a doubly acetylated derivative of bradykinin that was a by-product of the desired single acetylation reaction. The masses of the intense a_n -series fragmentation pattern indicated that

the second acetyl group was located on the side chain of the serine residue with the mass of the d_6 -fragment ion indicating loss of an acetyl group from the acetylated side chain of serine.

The series of x_n , $y_n + 2$, $z_n + 1$ backbone fragment ions with the positive charge located in the C-terminal region was also identified (when the amino acid on the C-terminal side of the cleavage site is proline, $x_n + 1$ and y_n ions are observed). In addition, six C-terminal fragmentations, consisting of three v_n (v_2 , v_4 , and v_5) and three w_n (w_3 , w_7 , and w_8) fragment ions, were also observed.

Negative Ion Spectra

The negative ion spectra of bradykinin and the two derivatives were also examined to determine whether any sequence-specific ions could be detected. The deprotonated molecular negative ion was observed for the parent molecule and the N-acetyl derivative but not for the O-methylated derivative. These spectra are shown in Figure 5. The absence of the negative molecular ion for the methyl ester is direct confirmation that deprotonation for bradykinin is occurring

Figure 5. Negative ion plasma desorption mass spectra of (a) N-acetylated bradykinin, (b) bradykinin, and (c) O-methylated bradykinin.

from the C-terminal carboxyl group. Although there is some evidence for loss of small neutral molecules from the negative molecular ion (possibly CO, N₂), no sequence-specific fragment ions could be detected in the negative ion spectrum. The intensity of the [M-H]⁻ ion for bradykinin is also only 2% of what was measured for the [M + H]⁺ ion.

Comparison with Fragmentation Patterns for Bradykinin Obtained by Other Methods

Part of the motivation for carrying out this study was to determine how the fragmentation pattern obtained for bradykinin by ²⁵²Cf-PDMS compared with what has been obtained by other methods. The ²⁵²Cf-PDMS method is markedly different from the other methods in two important respects. One of these is the time factor, and this is related to the experimental conditions at the ion source and the properties of the time-of-flight (TOF) method. When a bradykinin molecular ion is desorbed from the surface of the target, it is accelerated to its full energy in 108 ns in our spectrometer. If the molecular ion fragments after it leaves the acceleration region, the product species will contribute to the intensity of the molecular ion in the mass spectrum and not to the fragment ion spectrum. In order for a fragment ion to appear as a sharp peak in the mass spectrum at a TOF corresponding to its mass-to-charge ratio, the fragmentation must occur very close to the surface of the target and within 1 ns of excitation by the fission fragment. Thus, the fragmentation pathways available in ²⁵²Cf-PDMS under these experimental conditions are restricted to only those that occur on a subnanosecond time scale. This time domain is quite different from the microsecond to millisecond sampling times that prevail in a FAB source, collision cells, and multisector instruments.

The other major difference is the method of excitation of the molecule to a level where fragmentation occurs. In ²⁵²Cf-PDMS, the energy density in the region around the fission track is equivalent to temperatures between 5000 and 10,000 K [21]. High fluxes of photons and electrons are ejected from the fission track that can induce molecular excitation possibly to energy regimes not accessible by using other excitation methods. Direct electronic excitation of a molecule by a fission fragment is also possible because of the fission fragment's high atomic charge and velocity. The mechanisms of molecular excitation in ²⁵²Cf-PDMS are very complex and quite different from what occurs in a FAB source, a collision cell, or by laser photon excitation. With these differences in the time and energy domain in mind, we can now compare our results with what has been reported in the literature using other methods.

The first reported mass analysis of the ions from bradykinin produced in a FAB source clearly revealed the presence of fragment ions formed by metastable decay of the protonated molecular ion, but much of

the spectrum in the fragmentation ion region was obscured by the high density of background peaks [12]. An improved spectrum was published a year later by Martin et al. [22] that showed the metastable ion fragmentation pattern more clearly. Only a few of the sequence ions were observed, and those were centered around the phenylalanine residue with the dominant ion being the c₅ + 2 species. This pattern, when compared to what we obtained by ²⁵²Cf-PDMS, clearly shows that the excitation energy for bradykinin is different than what occurs in a FAB source. Recently, a prominent long-lived metastable ion fragmentation pathway was reported for bradykinin involving loss of the C-terminal arginine residue followed by rearrangement to retain one of the C-terminal carbonyl oxygens [13], giving rise to an ion at *m/z* 905 (904 u monoisotopic mass). We searched for this fragment ion in the ²⁵²Cf-PD mass spectrum but were unable to detect it. Two other fragment ions (y₈ and c₈ + 2) in the same mass region (*m/z* 905, 904) have been identified as components of the peak observed in the ²⁵²Cf-PD spectrum at *m/z* 903.95 (Figure 4b) from the mass shifts observed in the derivation studies. This fragment ion would have been a component of the *m/z* 904 peak in the mass spectrum of the bradykinin methyl ester (Figure 4c). However, the intensity of the observed peak is consistent with that observed for the C₈ + 2 fragment ion in the spectrum for the N-acetyl derivative (Figure 4a). We conclude that this rearrangement fragmentation pathway does not occur within the nanosecond decay time window of ²⁵²Cf-PD desorption/ionization.

Laser desorption has also been used to obtain metastable ion spectra of bradykinin [23]. The fragment ions observed are associated with backbone cleavage of the peptide. No side chain cleavages were detected. A peculiar feature of the spectrum was the absence of the molecular ion, which may indicate that the fragment ions observed may actually have been due to photolysis of the peptide in the solid state. This process can occur when a location on the surface of the sample is excited by multiple laser pulses [24].

The comparison of the metastable FAB mass spectra of bradykinin with the metastable ²⁵²Cf-PD mass spectra clearly indicates that the degree of internal excitation of bradykinin is much higher for ²⁵²Cf-PDMS. The appearance of an abundance of side-chain fragment ions (i.e., d_n, v_n, w_n) is a clear indication that this is indeed the case.

The most successful means to date for inducing excitation in bradykinin to dissociative states that give abundant sequence-specific ions has been CID. Previous studies have focused on two different energy regimes that are attained by exciting ions with low (15-100 eV) and high (> 5 keV) kinetic energy collisions. Poulter and Taylor [14] have recently made a comparison of the collision-induced fragmentation processes for the two energy regimes and have concluded that only at high collision energy are the

charge-remote site fragmentations and side-chain fragmentations of bradykinin observed. Both the charge-remote site and side-chain fragmentations are observed in the ^{252}Cf -PDMS of bradykinin and in FAB followed by high energy CID. The amino acids that generate d_n , v_n , and w_n fragment ions by FABCID (high energy) [25, 26] are also present in the ^{252}Cf -PD mass spectrum of bradykinin. We conclude that the excitation spectrum for bradykinin populated in ^{252}Cf -PDMS is close to that achieved by high energy collisions with He gas.

Concluding Remarks

The recent studies of Vorst et al. [10] and Buko and Sarin [11], which stimulated this current study, have demonstrated that ^{252}Cf -PDMS, contrary to popular opinion, can generate sequence-specific fragment ions extending to as high as 5.5 kD. Even for a peptide this size, the fragmentation pathways associated with high energy excitation processes are clearly evident. At this stage of development, it appears that the metastable ion spectrum generated by ^{252}Cf -PDMS has the *potential* for being competitive with the prevailing tandem mass spectrometry-based methodologies for sequencing peptides. Some of the problems associated with the metastable ion spectrum obtained by FAB (high level of chemical noise, low excitation energies) are not present in ^{252}Cf -PDMS. The clear advantage of the tandem mass spectrometry-based methodologies is the ability to mass select the molecular ion prior to excitation/dissociation, which removes many of the ambiguities that arise in the analysis of a metastable ion spectrum. However, these ambiguities can be minimized by careful purification techniques such as the use of microbore HPLC just prior to sample preparation for mass analysis and the use of mass-shift derivatizations to distinguish N-terminal and C-terminal fragment ions unambiguously. Finally, before ^{252}Cf -PDMS can be seriously considered as a viable alternative to existing methodologies, the mass resolution must be greatly improved, preferably to the point where mass measurements at the parts per million level of accuracy are possible [27].

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